AN OUTBREAK OF ASEP TIC MENINGITIS ASSOCIATED WITH ECHO VIRUS TYPE 4

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The group of enteric cytopathogenic human orphan (ECHO) viruses, described in 1955 by the Committee on the ECHO Viruses,1 at present comprises 19 types. Some of these have been obtained from the stools of healthy children;2-3 a number have been isolated from the stools of patients with the clinical syndrome of aseptic or non-bacterial meningitis, in the absence of additional evidence of an aetiological relationship to the disease;4 and several have been shown to be the probable causes of illness in man.

ECHO virus type 2 has been isolated from the spinal cord of a fatal case of bulbo-spinal paralysis resembling poliomyelitis.5, 6 Several investigators have recovered type 6 from the alimentary tracts of patients with aseptic meningitis,7-10 a rise in neutralizing antibody occurring in most of the cases. Type 9 has been associated with outbreaks of aseptic meningitis in which a number of patients developed a rash on the face, trunk and limbs, virus being recovered from cerebrospinal fluid11-13 as well as from the throat and from stools,14-16 while a rise in neutralizing antibody has been demonstrated. Type 4 has been described in association with an epidemic of aseptic meningitis in Marshalltown, Iowa, in 1955, the virus being recovered from 21 of 57 stools tested and from 2 of 7 throat washings. One cerebrospinal fluid was tested and found to be negative.17, 18

The present paper describes the isolation of ECHO virus type 4 from 10 of 18 children investigated during an outbreak of aseptic meningitis in an institution in Johannesburg, the virus being found in the cerebrospinal fluids of 7 patients and in the stools of 3.

Clinical Features

In the first 3 months of 1957 a high proportion of children in the Johannesburg Children's Home developed a syndrome characterized by pyrexia, severe frontal headache, vomiting and neck stiffness. During the earlier half of January all the children were absent on vacation with their relatives or friends. On 16 January 2 siblings returned, one of them becoming ill 5 days later. On 24 January the rest of the children returned, and within 3 days 2 developed a similar illness. In the succeeding 6 weeks, 58 out of a total of 121 children had become affected. The incubation period would appear to be short, 3 days having elapsed between the first and the next 2 cases, while during the following 9 days 38 other children developed the syndrome.

The Home was divided into 2 units, one for 30 infants and the other for 91 older children. These groups were not in direct contact with each other, the only links being the kitchen and the adult stair. The older children first became involved, and a period of 20 days elapsed before the infants were affected. During this time an adult nurse developed the illness, and it is possible that she conveyed the infection to the infants. Arthropod transmission was at first considered, and some mosquitoes were inoculated into tissue cultures without success. The subsequent recovery of virus from stools and the pattern of incidence suggest that a person-to-person transmission was operative.

The onset of illness was usually sudden. Pyrexia of 100-102°F and severe frontal headache were common, while nausea and vomiting occurred in more than half the cases. Neck and back stiffness, with or without a positive Kernig's sign, was present in the more severe cases, but tendon reflexes were normal and weakness was absent. Muscle tenderness was not a prominent feature, but was occasionally observed. Some children complained of photophobia or painful eyes, and suffusion of the conjunctivae was noted in a few. The illness was mild in the majority of cases, persisting for a few
days only. A notable feature was the tendency to relapse, with identical clinical findings, observed in 10 children; 5-10 days elapsed between recrudescences; one child had 2 relapses while another had 3.

Sixteen children were admitted to the Johannesburg Fever Hospital for further investigation. Two of these showed, in addition to the common signs, moderately enlarged spleen and tender cervical, axillary and inguinal lymph nodes. One had a palpable liver, with mild disturbance of hepatic function as reflected in biochemical serum tests. Blood counts on these 2 cases showed predominance of lymphocytes, with atypical forms present; but the Paul-Bunnell tests were negative. Neck and back stiffness was present, and in both cases there was pleocytosis in the cerebrospinal fluid, virus being obtained from the fluid of one (J.S.).

**Laboratory Investigations**

Cerebrospinal fluids from 18 cases, taken shortly after admission to hospital, usually between the 1st and 3rd days of illness, were examined. In 2 instances the fluid was normal, but one of these patients (F.B.) was carrying virus in the stool. In the other 16 fluids pleocytosis was the chief abnormality. Lymphocytes occurred in a range of 5-150 per c.mm. and predominated in 10 fluids; while polymorphonuclear leucocytes occurred in a range of 0 to 610 per c.mm., and predominated in 6. In 5 fluids the protein values (34 to 55 mg. per 100 ml.) were slightly above the upper limit of normal (30 mg. per 100 ml.). Sugar and chloride estimations fell within the normal ranges, and no bacteria could be detected.

A polymorphonuclear leucocytosis (10,000-19,000 per c.mm.) was found in the blood of 8 of 17 patients, but the counts were otherwise not markedly altered, with the exception of the 2 cases showing atypical lymphocytes, one of whom (J.S.) had a lymphocytosis of 22,700 per c.mm.

Other routine laboratory investigations included the Paul-Bunnell test and complement-fixation tests for leptospira, rickettsiae, and the viruses of herpes simplex, mumps and lymphocytic choriomeningitis; all of which were negative.

**Virus ISOLATION**

Monkey kidney roller tube cultures, prepared from fresh trypsinized renal epithelium of the vervet monkey *Cercopithecus aethiops pygerythrus* were grown in a medium containing 0.5-5% lactalbumin hydrolysate in Hanks' salt solution and 5% horse serum. Just before inoculation the fluid was changed, with the omission of serum; and every 4-5 days the medium was renewed, with the addition of 0.5% horse serum.

Human amnion roller tube cultures, prepared from fresh trypsinized membranes, were grown in a medium consisting of Parker medium no. 199 with 20% human serum. Before inoculation and at subsequent fluid renewals the medium was changed to Parker no. 199 or 0.5% lactalbumin hydrolysate in Hanks' salt solution with 0.5-2% horse serum.

Strain HeLa human malignant epithelial cells, kept in continuous cultivation since 1954, when they were received from Dr. G. O. Gey of Baltimore, were grown in a medium consisting of Parker no. 199 with 0.025% Difco yeastolate, 0.05% lactalbumin hydrolysate, and 30% human serum. Before inoculation and at subsequent fluid changes they received a medium consisting of Parker no. 199 with 2% horse serum.

Specimens from 18 patients in the acute phase of illness were tested in tissue cultures for the presence of virus as soon after collection as possible, in a number of cases less than an hour elapsing before inoculation. All tissue cultures were inoculated between 36-37°C.

**Blood.** 0.3-0.5 ml. samples of whole blood from 3 patients were inoculated into kidney cultures, which were then observed for 24-33 days without cytopathic changes becoming apparent. Included here were patients F.B., who had virus in the stool, and C.M., whose cerebrospinal fluid contained virus.

**Throat Swabs.** Swabs from 5 patients were agitated in 1-2 ml. of Hanks' salt solution containing antibiotics, and the fluids were inoculated into kidney cultures, which were observed for 27-33 days without cytopathic changes being noted. Of these patients, C.M. had virus in the cerebrospinal fluid, while M.B. and C.H. carried virus in their stools.

**Rectal Swabs.** Rectal swabs were agitated in 1-2 ml. of Hanks' solution containing antibiotics, and the fluid was centrifuged at 3,000 r.p.m. for 20 minutes. Supernatant fluid amounts into each of 3 kidney culture tubes. Swabs from 7 patients were examined, and from 3 (F.B., M.B., C.H.) virus was obtained. Negative cultures were observed for 27-33 days, and there was no evidence of virus on blind passage.

**Cerebrospinal Fluid.** Kidney cultures were inoculated with cerebrospinal fluids from 16 children, 0.15-0.25 ml. being put into each of 3 tubes for each patient, and virus was recovered from 7 (B.C., S.F., Y.F., J.G., C.M., A.S. and J.S.). Two patients whose cerebrospinal fluids were negative had virus in the stool (F.B., M.B.). The recovery of virus from the fluid of C.M. was successfully repeated, re-inoculation being attempted in the other cases.

**Viral behaviour in tissue culture**

Cytopathic effects in kidney cultures became apparent in primary isolations on the 5th to 8th day, and there was a prompt passage of undiluted fluid resulting in the complete destruction of cultures within 4 days. In a number of instances harvested culture fluid was stored for 5-9 weeks at -20°C, with a resultant marked drop in infectivity until further passage restored the titre. Titrations of strains showed levels ranging from 10^4 to 10^7 TCID50 (doses infective for 50% of the tissue cultures) per 0.1 ml.

No cytopathic changes were seen over periods of 11-16 days when human amnion cultures were inoculated with 10^6 and 10^6 TCID50 of strain C.M., although kidney cultures inoculated in parallel were completely destroyed by the 4th day. Strain A.S. was also inoculated into amnion and kidney cultures in parallel, the former showing no changes over 20 days, while the kidney cultures were completely destroyed by the 6th day. Rectal swab suspensions and cerebrospinal fluids which proved to be positive for virus in kidney cultures were inoculated in parallel into amnion cultures during the original isolation attempts, with negative results.

HeLa cell cultures were inoculated with the C.M. strain on 2 occasions and observed for 7 and 9 days without cytopathic effect. Kidney cultures inoculated in parallel showed complete destruction by the 4th day.

Monkey kidney cover-slip cultures lying free in roller tubes were inoculated with 6 strains of virus, fixed with Bouin's solution, 24-27 hours later. Cytopathetic changes included eosinophilic staining of the nucleus, and the appearance of a paranuclear zone which stained a deeper red than the rest of the cytoplasm. Later, the nucleus became folded or scrolled, which now stained less intensely than the remaining cytoplasm. Finally the cells became shrunken and rounded, with dense cytoplasm and small dark nuclei. No inclusions were observed.

**Virus IDENTIFICATION**

(a) Neutralization Tests

Strain C.M. was chosen as the prototype, and was tested against immune sera for ECHO virus types 1-16, 18 and 19 which had been kindly supplied by Dr. A. B. Sabin of Cincinnati and Dr. J. L. Melnick of New Haven. Antiserum for type 17 was not available. Three rabbit sera prepared in our laboratories for the 3 types of poliovirus were also included. An estimated challenge dose of 100 TCID50 was mixed with an equal volume of inactivated serum diluted 1 in 10 to give a final serum dilution of 1 in 20, for inoculation into each of 3 kidney cultures after being held at 4°C for 1 hour. Tubes read at the 8th day showed complete destruction except where sera for ECHO types 19 and Polio types 1, 2 and 3 had been used. The protection by sera for ECHO types 4 and 18 and for Polio 1 and 3 was complete;
ECHO type 19 serum protected 1 tube fully and 2 tubes partially; while Polio type 2 serum protected 2 tubes fully and 1 tube partially.

Neutralization by the protecting sera was re-tested, with the use of a challenge of 1,000 TCID50 as measured in a parallel titration, and the cultures were observed for 7 days. Control cultures inoculated with the challenge dose only were entirely destroyed by the 5th day; complete protection was given by ECHO type 4 serum; while a slow break-through occurred where sera for ECHO types 18 and 19, and for Polio types 1, 2 and 3 were used.

The ECHO and Polio sera which gave partial protection against strain C.M. were all derived from rabbits, and the possibility exists that a neutralizing factor resistant to inactivation at 56°C for 30 minutes exists in strains of ECHO virus type 4 may be encountered in rabbits.

The 10 strains isolated were then tested with a monkey antiserum to ECHO type 4 produced under the auspices of the Committee for ECHO viruses and sent to us by Dr. H. A. Wener of Kansas City. The dried serum was diluted 1 in 5, inactivated at 56°C for 30 minutes, cooled, and mixed with an equal volume of virus suspension estimated to contain 100 TCID50 per 0·1 ml., giving a final serum dilution of 1 in 10. This was held at 4°C for 1 hour, and then 0·2 ml. amounts of each serum-virus mixture were inoculated into each of 4 kidney culture tubes. Uninoculated control tubes were set up, together with 3 tubes inoculated with the virus challenge dose only, for each strain tested. The cultures were incubated at 36°C, and were read on the 3rd and subsequent days. Neutralization was considered to have occurred when all control tubes inoculated with virus only showed cytopathic effect involving the entire culture and no change was present in the tubes inoculated with serum-virus mixture. Further readings were taken at least 24 hours later to check that protection had been maintained. By these criteria, all the 10 strains were neutralized by the ECHO type 4 serum.

At the same time, the serum in a final dilution of 1 in 50 was tested against each strain. In every instance it failed to protect at this dilution. The serum was stated to have a neutralizing titre of 30-75 against 32-200 TCID50, which is conspicuously lower than that of sera produced by the same workers against the rest of the first 13 ECHO viruses. At a final reading of the neutralization test on the 7th day, 6 strains showed a late break-through in occasional tubes, protection in all 4 tubes persisting for the other 4 viruses. It is felt that the failure of the 1 in 50 serum dilution and the partial late break-through in some instances was due to the initial low titre of the antiserum.

(b) Filtration

Tissue culture fluid containing strain C.M. was filtered under 2-3 lb. pressure through a membrane filter having an estimated maximum pore diameter of 450 millimicra and an average pore diameter of 275 millimicra. Both pre- and post-filtration fluids produced complete destruction of kidney cultures within 3 days. The culture fluid was immediately followed by a suspension of Serratia marcescens in buffered saline under pressure of 5 lb. The pre-filtration suspension produced a profuse growth on coagulated serum medium within 48 hours, but no bacteria could be detected in the post-filtration fluid.

(c) Ether

Tissue culture fluid containing strain C.M. was centrifuged at 3,000 r.p.m. for 30 minutes and the supernatant fluid was divided into two aliquots. To one, ether was added up to a quarter of the total volume, while the other sample was held as a control. Both were kept at 4°C for 24 hours, and were well shaken several times during that period. Titration after 24 hours showed that the unetherized sample contained over 10⁸ TCID50 per 0·1 ml., while the etherized sample contained 10⁶ per 0·1 ml.

(d) Mice

Litters, each comprising 6 infant white mice approximately 24 hours old, were inoculated with undiluted tissue culture fluid. Two litters were inoculated by the combined intraperitoneal-subcutaneous route, and one litter by the intracerebral route, with the other 8 remaining controls. All signs of disease were noted during the observation period of 21 days, and histological examination of one mouse from each litter taken on the 10th day showed no lesions. Strain F.B. was also inoculated into similar litters by the same routes, and no signs of disease were detected.

Eight 4-5-week-old white mice were inoculated intracerebrally with strain C.M., and showed no signs of illness over 21 days. Tissue culture controls of all inocula demonstrated the presence of virus.

(e) Rabbits

Two rabbits given 4 intravenous inoculations of undiluted tissue culture fluid containing strain C.M. for the purpose of producing immune serum showed no signs of illness.

Neutralization with paired sera

In Table I are given the results of a neutralization test using 316 TCID50 of strain C.M. against acute- and convalescent-

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<thead>
<tr>
<th>Serum (final dilution 1 in 2)</th>
<th>Tubes showing cytopathic changes on days</th>
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<tbody>
<tr>
<td>M.B. February 6</td>
<td>3</td>
</tr>
<tr>
<td>March 12</td>
<td>3</td>
</tr>
<tr>
<td>V.G. February 18</td>
<td>3</td>
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<tr>
<td>March 9</td>
<td>3</td>
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<tr>
<td>C.H. February 6</td>
<td>3</td>
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<tr>
<td>March 12</td>
<td>3</td>
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<tr>
<td>A.L. February 6</td>
<td>3</td>
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<tr>
<td>March 12</td>
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It is clear that ECHO virus type 4 is a poor antigen for the production of neutralizing antibodies. This is shown not only by the low titre of the monkey antiserum, but also by the findings of Chin et al., who noted that in their series of patients the rise of antibody was slow, did not exceed a low level, and was absent in 2 cases with aseptic meningitis. It is therefore probable that evidence of neutralizing antibody rise between paired sera will be difficult to demonstrate when clinical cases of ECHO type 4 infections are encountered. This would seem to eliminate one of the most valuable proofs of aetiology, and some other indication of immunity or a more sensitive test may have to be sought. Poor antibody formation may account for the striking incidence of relapses, and another immunity mechanism may determine recovery particularly where the course of the disease is short.

In the present series of cases, isolation of virus from the cerebrospinal fluids of patients with the syndrome of aseptic meningitis should suffice to confirm the causal relationship, in the absence of marked antibody rise.

Only 7 rectal swabs were examined in the present investigation, 3 being positive. It is felt that if stools had been taken, a higher isolation rate would have been achieved, since another unit in these Laboratories at the same time was investigating stools from the Fever Hospital for poliovirus, and recovered non-polio viruses not yet further characterized from the stools of 3 patients in our series who had virus in.
their cerebrospinal fluids. Faecal contamination would account readily for the manner in which infection spread through the institution in which the outbreak occurred.

The selective affinity of this virus for monkey kidney cells contrasts with the behaviour of ECHO type 9, which grows better on human amnion and is pathogenic for infant mice. This serves to demonstrate that the successful isolation of viruses necessitates the use of a variety of tissues.

SUMMARY

During an outbreak of aseptic meningitis in a children's institution in Johannesburg in 1957, a virus resembling ECHO virus type 4 was isolated from the cerebrospinal fluids of 7 patients and from the rectal swabs of 3 other patients.

A marked rise in the neutralizing antibody content of convalescent-phase sera tested did not occur, and it is suggested that this might account for the relapses noted in the outbreak.

The virus was isolated in tissue cultures of monkey kidney, and did not produce cytopathic changes in cultures of human amnion or HeLa cells.

We wish to thank Dr. J. W. Scott-Millar, Medical Officer of Health for Johannesburg, for allowing us to investigate these cases; Dr. A. L. Jackson, Physician-in-Charge of the Fever Hospital, for permission to consult records; and Dr. R. Brueckner and Dr. E. Chesler for collecting specimens. The cooperation of the Matron and staff of the Johannesburg Children's Home in facilitating the investigations is gratefully acknowledged. We also thank Dr. J. H. S. Gear, Director of the Poliomyelitis Research Laboratories, for the encouragement he has given to us during this study.

REFERENCES


BOOKS RECEIVED: BOEKE ONTVANG


